Joint associations of blood plasma proteins with overwinter survival of a large mammal

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Abstract

In many wild animal populations, hosts are at risk of parasites and malnutrition and resource costs of defence may be difficult to afford. We postulate that proteins, important in homeostasis and immunity, play a complex but central role in condition-dependence and resource costs of mammalian immune defense. To test this, we measured plasma concentrations of albumin, total proteins. self-reactive antibodies and parasite-specific IgG in female Soay sheep. Using a Principal Component Analysis, we found a new metric of condition reflecting individual variation in acquisition, assimilation and/or recycling of plasma proteins that predicted overwinter survival. Controlling for this metric, an age-dependent trade-off between antibody titres and protein reserves emerged, indicating costs of mounting an antibody response: younger individuals survived best when prioritizing immunity while older individuals fared better when maintaining high protein nutritional plane. These findings suggest fascinating roles for protein acquisition and allocation in influencing survival in wild animal populations.

Introduction

Individuals vary greatly in the strength, specificity and efficacy of immune responses triggered by infection (Frank 2002). The maintenance of this variability by natural selection can be due to many factors (Lazzaro & Little 2009) but theory suggests that the magnitude and type of costs associated with the immune response play an important role (Boots *et al.* 2009). Variable immune defenses may thus reflect the balance of costs and benefits of immunity at the individual level (Viney *et al.* 2005). For instance, misguided or overly strong immune responses may result in immunopathology that reduces host fitness (Graham *et al.* 2005) and alters evolutionarily stable strategies for the rate at which hosts recover from infection (Cressler *et al.* 2015). In parallel, hosts pay resource costs of defense when they experience a trade-off in the allocation of limited resources between the immune response and other physiologically demanding life history traits (Lochmiller & Deerenberg 2000). Indeed, in animals in both the laboratory and the wild, the production of an immune response following exposure to novel antigens can increase metabolic rates (e.g. Demas *et al.* 1997; Mendes *et al.* 2006) and reduce the energy allocated to other costly activities (e.g. Råberg *et al.* 2000; Canale & Henry 2011). However, biomarkers of nutritional physiology and immunology have never, to our knowledge, been tracked simultaneously nor used to assess resource costs of immunity in a wild animal. Such an analysis would provide insights into how different strategies of allocation of limited resources arise in hosts facing parasite and nutritional challenges.

Many host taxa are reliant on proteins for both homeostasis and immunity, and proteins are thus likely to be at the center of resource costs of immunity. Albumin and total immunoglobulin G (IgG), in particular, represent 80-90% of the mammalian blood plasma protein pool at any given time and albumin alone represents a very high fraction (Sand *et al.* 2015). These proteins also exhibit dynamic variation in their relative concentrations as they fulfill vital physiological roles. Albumin synthesis in the liver is very plastic, for example, with the potential for rapid decreases in response to fasting (Peters 1985) or inflammation, followed by rebounds when protein nutrition is restored (Morgan & Peters 1971). Albumin

66 is instrumental in the maintenance of osmotic pressure, the mechanism by which fluids are kept within the
67 blood circulation (Mazzaferro *et al.* 2002). IgGs, by contrast, are among the main effectors of the humoral
68 immune response (Manz *et al.* 2005). Albumin and IgG share the particularity of being the longest lived
69 of all plasma proteins, with half-lives of 19-21 days in humans (Sand *et al.* 2015) and around 14 days in
70 sheep (Fell *et al.* 1969; Smith *et al.* 1976). These long half-lives arise because both are protected from
71 intracellular catabolism pathways through recycling mechanisms involving the neonatal Fc receptor FcRn
72 (Pyzik *et al.* 2015): both IgG and albumin can bind this receptor at the acidic pH of endosomes and be
73 returned to the circulatory system unscathed. While the role of FcRn in recycling both IgG (Junghans &
74 Anderson 1996) and albumin (Chaudhury *et al.* 2003) has been mostly studied in humans, similar roles
75 for FcRn in maternal immunity and IgG metabolism have been shown in ruminants, although whether
76 they also extend to albumin remains to be determined (Cervenak & Kacskovics 2009).

77 Here, we propose that the abundance, physiological importance and shared recycling pathways of
78 albumin and IgG make these molecules likely to be central to quantifying both host condition and any
79 protein costs of humoral defense. For instance, individuals that maintain high albumin and
80 immunoglobulin concentrations may be better than others at protein acquisition (for instance through
81 better foraging and/or selection of grazing grounds) but are potentially also better at recycling IgG and
82 albumin. Such a recycling strategy may promote individual "condition" (for the purpose of this study,
83 defined as an individual's ability to acquire, assimilate and/or maintain circulating protein concentrations)
84 and could prove particularly beneficial during periods of resource scarcity and malnutrition. Independent
85 of varied condition, individuals may also experience a trade-off between investment in albumin and
86 immunoglobulins (arguably representing a protein cost of immunity): nutrients used to produce one type
87 of protein would not be available to produce the other one, especially when resources are scarce. Such
88 trade-offs can be obscured by other quantitative traits (Pemberton 2010) and might only become apparent
89 after differences in nutrient acquisition, assimilation and/or maintenance are taken into account (van
90 Noordwijk & de Jong 1986).

Proteins synergise with fats to provide crucial nutrients for mammals during times of scarcity. In wild Northern ungulates, winter represents an important energetic bottleneck because of the low nitrogen content of winter forage (Owen-Smith 2002). Animals must therefore rely on resources stored during the summer and autumn grazing periods and the levels of body protein and fat attained may be more important to survival than total body weights (Adamczewski *et al.* 1987). Fat appears to be the most important energy store (Parker *et al.* 2009), but the importance of protein catabolism as an alternative energetic resource increases with the severity of winter. Indeed, proteins appear to be used at a faster rate when fat stores approach depletion in wild ungulates exposed to harsh winter conditions (in caribou: Adamczewski *et al.* 1987; in reindeer and caribou: Barboza & Parker 2008; in mule deer: Monteith *et al.* 2013). Selection of favourable summer grazing areas promotes pre-winter fat accumulation (Long *et al.* 2016). However, summer nitrogen availability represents a bigger constraint in some systems (McArt *et al.* 2009),especially in ungulate populations experiencing high densities which reduce the quality of the forage (Herfindal *et al.* 2006), and increase immunological demands (Downs *et al.* 2015). In such systems, albumin and IgG are likely to be currencies for host condition and to mediate costs of immunity.

We elected to investigate our hypotheses in ungulates experiencing high densities in times of scarcity: the Soay sheep of St. Kilda, Scotland (Clutton-Brock & Pemberton 2004). The dynamic of this population is marked by the recurrence of high mortality winters, or "crashes," during which up to 70% of the population dies in late winter (Clutton-Brock *et al.* 1992; Grenfell *et al.* 1992; Coulson *et al.* 2001). As in many wild systems, weight and age have been associated with overwinter survival: heavier and prime-aged individuals are more likely to survive than lighter and post-prime-aged sheep (Clutton-Brock *et al.* 1997; Coulson *et al.* 2001). Gastrointestinal nematodes, including *Teladorsagia circumcincta*, negatively affect survival, particularly in young individuals (Gulland 1992; Coltman *et al.* 1999; Hayward *et al.* 2011), while antibodies of several specificities, most importantly IgG against larval *T. circumcincta,* are positive predictors of adult survival (Graham *et al.* 2010; Nussey *et al.* 2014; Watson *et al.* Submitted). However, the contributions of varied nutritional plane or individual condition to varied antibody titers are

unknown, and the role of nutrition has been studied during only one crash: Gulland (1992) collected gross pathological evidence consistent with severe malnutrition at the time of death and showed a marked decline of several physiological markers of protein nutrition, including albumin, in surviving individuals. Here, we investigate how summer protein and antibody concentrations were associated with subsequent survival of adult female sheep during three high mortality winters. We expect the combination of markers to jointly reflect condition (i.e. the ability to acquire, assimilate and maintain plasmatic protein levels) and have a positive effect on overwinter survival. Further, we hypothesize that once varied condition is taken into account, our analysis may reveal a resource allocation trade-off between antibodies and maintaining nutritional plane that would further impact survival probabilities.

Materials and methods

The population of Soay sheep on Hirta, in the St. Kilda archipelago (Scotland), has been unmanaged since sheep were introduced from the nearby island of Soay in the 1930s, and before that experienced minimal management on the island of Soay for millennia. The subpopulation inhabiting the Village Bay area has been monitored since 1985 (Clutton-Brock & Pemberton 2004). Each year, most lambs are caught and individually ear-tagged within their first week of life. Each August, as many individuals as possible are caught, weighed, measured and sampled for blood and feces. Blood samples are immediately stored at 4°C. Within 24 hours of collection, plasma is separated by centrifugation (10 000g, 10 minutes), aliquoted and stored at -20°C. The current study centers on three overwinter mortality events (population 'crashes') that occurred during the winters of 1998-1999, 2004-2005 and 2011-2012.

We focus on female sheep that were at least 2 years of age when sampled in August prior to a crash. This age and sex group represents the most abundant host type in the population. Concentrations of Anti-Nuclear Antibodies (ANA) and IgG directed against L3 stage larval antigens of *T. circumcincta* were already available for samples collected during the August preceding the first two crashes studied here (Graham *et al.* 2010; Nussey *et al.* 2014). Data on *T. circumcincta*-specific IgG from August 2011 were

141 also available (Watson *et al.* submitted). In order to measure ANA for the 2011 samples, we modified a
142 commercial kit developed to measure ANAs in humans (Orgentec ANA detect, Orgentec, Germany). This
143 kit contains the same antigens used to measure ANA previously and we followed the modified protocol
144 for sheep (Graham *et al.* 2010).

145 In plasma collected in the August prior to the three crashes, we assessed the protein nutritional status of
146 individuals by measuring concentrations of albumin and total proteins (TP; used to correct for varied
147 overall fluid concentrations because sample volume and field constraints made it impossible for us to
148 directly measure plasma colloid osmotic pressure or plasma osmotic concentration in this study). The
149 assays were first validated for small sample volumes using domestic sheep plasma samples, so that the
150 final volume of sample required for duplicate assays was 10 µL. Total proteins were measured using a
151 classical colorimetric assay based on the Coomassie assay. Briefly, plasma samples were diluted 1:100 in
152 Milli-Q water and 10µL of diluted sample was added to each well in a 96-well plate. Subsequently,
153 300µL of Coomassie reagent (Coomassie Plus Reagent, Thermo Scientific, USA) were added to each
154 well. Plates were then placed in a spectrophotometer, shaken for 45 seconds and incubated for 10 minutes
155 at 23°C. After incubation, optical densities (OD) were read at 595nm. Another colorimetric assay was
156 used to measure plasma albumin. Plasma samples were diluted 1:4 in Milli-Q water and 5µL of diluted
157 sample was added to each well in a 96-well plate. In each well, 200µL of BromoCresolGreen reagent
158 (Quantichrom BCG albumin assay, BioAssay Systems, USA) was carefully added. Plates were then
159 placed in a spectrophotometer, shaken to eliminate bubbles and incubated for 5 minutes at 23°C. After
160 incubation, OD values were read at 620nm. For both albumin and total protein assays, all samples were
161 run in duplicate. In addition, 8 ovine albumin standards of known concentrations were run in duplicate on
162 each plate, allowing plate-specific standard curves to be used to calculate the concentration of a sample
163 based on the mean measured OD across the duplicate wells. Standard curves were fitted to the average of
164 the 2 standards on each plate separately using a 4 parameter logistic regression with the package “drc” in

R version 3.2.1 (Ritz & Streibig 2005). OD values for all samples fell in the intermediate, quasi-linear, portion of their respective standard curves.

We analyzed data on a total of 285 females for which all 4 markers of interest (ANA, anti-*T. circumcincta* IgG, total proteins and albumin) were available. We then evaluated their separate and combined power to predict subsequent survival, using Generalized Linear Mixed Models (GLMM) as described below. Next, we performed a Principal Component Analysis of the physiological data using the package 'FactoMineR' (Lê *et al.* 2008) in R 3.2.1. We elected to include total proteins in the PCA not only to correct for possible variations in plasmatic concentration due to varied hydration levels (e.g. Horowitz & Samueloff 1979) but also because it contains additional information neither included in albumin or the specific immunoglobulins we measured. Our aim was not reduction of dimensionality per se, but instead to parse the joint as well as distinct effects of nutrition and immunoglobulins upon survival. This approach allows the conversion of our originally weakly correlated variables into orthogonal (i.e. uncorrelated) predictors without loss of variance, which can then be used in further statistical analyses (e.g. Stoddard *et al.* 1988; Arnqvist & Tuda 2010). We focused our analysis on the first two principal components (PC), which accounted for the majority of the variance (Table 1). We assessed associations of PC1 and PC2 scores with August body weight, August body weight conditioned on hind leg length, and reproductive success (i.e., having given birth or not) in the preceding spring. We then determined how PC1 and PC2 were associated with overwinter survival. Using the ratio of albumin to total proteins to correct for variations in hydration results in similar principal components and the associations with survival we report are also recovered when using this other approach.

The long term study of the Soay sheep population of St. Kilda (see Clutton-Brock & Pemberton 2004) means that the fate of most tagged individuals on the island is known. In addition, various well-described predictors of overwinter survival of individuals, such as age and August weight, are available. In most cases, strongyle fecal egg counts (FEC), an estimate of the August burden of gastrointestinal nematodes, are also available (see Craig *et al.* 2006). We used GLMMs to estimate the association between

overwinter survival and these various predictors, in addition to the raw and decorrelated marker data. The year of capture was used as a random effect, though our inferences were robust to year fitted as a fixed effect. Too few individuals were observed on more than 1 occasion in this dataset (23 individuals in total; 20 between 1998 and 2004 and 3 between 2004 and 2011) to allow the fitting of a random effect of individual identity. In all models, body weight (mean-centered and scaled to unit variance) was added as a predictor of overwinter survival following previous work (e.g. Nussey *et al.* 2014). Furthermore, we assessed whether reproductive success the previous spring was predictive of subsequent crash survival. We also used age as a predictor of overwinter survival, either as a continuous variable or through age classes (following Coulson *et al.* 2001) by separating prime aged females (2-6 years old) from older females (7 years old and older). We then included raw markers or PC1 and PC2 and all two-way interactions as predictors of survival. The best model was selected based on Akaike's Information Criterion (AIC) values, with lower values indicating improved model fit. Models were run with the packages 'lme4' (Bates *et al.* 2015) and 'lmerTest' (Kuznetsova *et al.* 2015) in R version 3.2.1. Effects of factors were estimated using the package 'effects' (Fox 2003), and plots were obtained using Python 2.7 in Beaker notebooks.

Results

Within individual sheep, the correlation structure among the markers was complex (see Table S1), with albumin and IgG uncorrelated. Each of the four markers was significantly (or marginally non-significantly) associated with overwinter survival, either as a main effect or through an age-dependent interaction. The set of best models predicting survival (see Table S2) consistently included IgG and various combinations of the other three predictors, with or without interactions with age. Interestingly, no interactions among markers, including albumin with IgG, were retained in the set of best models (all

p>0.5). However, these analyses do not resolve whether the association of survival with IgG, for example, reflects variation in resource acquisition and allocation.

Consequently, we performed a Principal Component Analysis (Figure S1; Table 1). All markers loaded in the same direction on the first principal component (PC1) which accounted for almost 41% of the total variance. Interestingly, PC1 was independent of both weight (R^2=-0.003, p=0.76) and the residuals of a regression of weight on hindleg length (R^2=-0.003, p=0.92). Taken together, these results indicate that PC1 might represent a weight- and body size-independent marker of condition, potentially reflecting individual variation in acquisition, assimilation and/or recycling of proteins. The second principal component (PC2) accounted for over 30% of the total variance and suggested a resource trade-off between nutrition (TP, Albumin; positive loading) and immunity (ANA, anti-*T. circumcincta* IgG; negative loading): high values of PC2 represent a high investment in homeostasis and protein reserves while low values represent an investment skewed towards antibodies. As with PC1, we found no evidence for an association with weight (R^2=0.003, p=0.16) even after accounting for body size (R^2=0.001, p=0.25). PC1 and PC2 were not associated with reproductive success the preceding spring or with nematode burden (estimated as fecal egg count, or FEC; all p>0.5). Finally, PC3, accounting for 16% of the total variance, captured differences between ANA (loading positively) and anti-*T. circumcincta* IgG (loading negatively).

We next used PC1 and PC2 scores along with weight, age, recent reproduction, and parasite burden as predictors in GLMMs of overwinter survival. When we modeled categorical age classes (Table 2, upper row; prime-age versus older females (Table 2, upper row; prime-age versus older females, following Coulson *et al.* 2001; Ozgul *et al.* 2009), independent of effects of weight, PC1 was a significant positive predictor of survival (PC1: z = 4.02, p < 0.001; Figure 1). PC2 was also significant, through an interaction with age class (PC2*age class: z = -2.84, p = 0.004). In prime-age individuals (Figure 2, blue curve), overwinter survival decreased with increasing values of PC2, indicating that the probability of survival increased with increasing investment in immunoglobulins. Older individuals were instead more likely to

survive when maintaining albumin in relation to total proteins as indicated by the positive survival association of PC2 in that age class (Figure 2, green curve). When we performed the same analyses using age as a continuous variable (Table 2, bottom row), a comparable model (including the interaction) was selected as best, although the support for the interaction was less strong (PC2*age: $z = 1.98$; $p = 0.048$). Neither recent reproductive success nor August nematode fecal egg counts (FEC, used as log(FEC+1) to account for overdispersion) were significantly associated with survival (both $p > 0.5$).

Discussion

Given the widespread co-occurrence of malnutrition and infectious disease in natural populations worldwide (e.g. Koski & Scott 2001), understanding how individuals allocate limited resources to immunity and general homeostasis is critical. Here we report that, independent of body weight and immunoglobulins (Nussey *et al.* 2014; Watson *et al.* Submitted), additional variations in host survival is explained by protein reserves, particularly in the form of albumin. However the complex correlation structure between the different markers potentially reflects conflicting processes: variations in acquisition, assimilation and/or recycling would result in positive associations between albumin, antibodies and total proteins while differential allocation to albumin versus immunoglobulins would conversely result in negative associations. We thus further aimed at better parsing joint and distinct effects among these biomarkers using a PCA. We first found a novel, weight- and size-independent metric of individual condition that maximises individual survival. Further, a trade-off between protein nutritional plane and antibodies, which might otherwise have been obscured (van Noordwijk & de Jong 1986; Pemberton 2010), became apparent once the variation in resource acquisition, assimilation and/or maintenance was taken into account.

We found that the first principal component was a powerful predictor of survival, consistent with PC1 reflecting variation in individual abilities to acquire, assimilate and/or recycle proteins. Measuring other

aspects of nutrition such as leptin, a hormone related to fat metabolism and immunity (Brestoff & Artis 2015), or fatty acids (which are of particular importance in ruminants; Jiang *et al.* 2014), would provide further insight into whether PC1 is a general marker of condition or whether it specifically represents protein storage and metabolism. More generally, it would be of interest to investigate the relative contributions of micronutrients (e.g. Vitamin D, a predictor of Soay sheep fecundity; Handel *et al.* 2016) and macronutrients (such as protein) to individual condition and hence immune function, survival and reproduction across the full 30+ year time series of the Soay sheep study. Fat stores would be particularly interesting as these resources are primarily used to meet winter energetic demands (Parker *et al.* 2009). Similar to other ungulates (Adamczewski *et al.* 1987; Barboza & Parker 2008; Monteith *et al.* 2013), the protein reserves of Soay sheep may only come into play when fat accumulation has been hampered by high sheep densities and/or low quality browse. Collection of data on proteins as well as other physiological markers, in the Soay sheep and in other populations subjected to overwinter nutritional stress, would allow the assessment of the longer-term repeatability and cross-system generality of the associations we report here, and the relationship of proteins to other aspects of nutrition.

The positive effect size on survival of this PC1 was notably independent of, and similar in magnitude to, the effect of weight, a long recognized predictor of survival in Soay sheep (Clutton-Brock *et al.* 1997), indicating that they are equally important for overwinter survival. We interpret PC1 as a potential metric of "condition" that predicts individual robustness during times of resource scarcity. Marked seasons are common in Northern Europe, and Northern ungulates in general and Soay sheep in particular are faced with important energetic challenges. These seasons normally involve a pulse of plant growth during a favorable season and an extended period without plant growth. The extent of the resulting winter food shortage and loss of body condition for herbivores varies, for instance in relation to their population density (e.g. Hallett *et al.* 2004). Because of the low nitrogen content of winter forage (Owen-Smith 2002), robust liver physiology, including production and recycling of plasma albumin, appears particularly important to overwinter survival when mass starvation occurs (Gulland 1992). In other words,

when resource acquisition becomes near-impossible in winter, individual variation in assimilation or recycling could become paramount. Interestingly, FcRn is expressed in hepatocytes and can prevent leaking of both IgGs and albumin in the bile (Sand *et al.* 2015). Sheep with higher PC1 scores may thus be the ones that better maintain plasma proteins in the face of malnutrition, potentially through increased levels of FcRn expression. Further studies will be necessary to understand the potential physiological importance of protein recycling in this system and in other wild ungulate populations.

Furthermore, we found that, consistent with our predictions as well as theory (van Noordwijk & de Jong 1986), accounting for positive correlations among all markers (with PC1) revealed an allocation trade-off between albumin and total proteins on the one hand, versus antibodies on the other. High PC2 scores indicated individuals favoring maintenance of homeostasis and investment in protein reserves while low scores indicate individuals favoring antibodies. This trade-off accords with the hypothesized protein cost of immune responses (Lochmiller & Deerenberg 2000), and its consequences appear to be age-dependent: younger individuals survived population crashes better when investing more in parasite-specific IgG, while older individuals achieved their best survival via high albumin and total protein concentrations. This potentially reflects differential importance of nematode tolerance (Hayward *et al.* 2014b) across the lifespan, driven by the nutritional status early in the yearly cycle (Houdijk *et al.* 2001). Alternatively, older individuals with senescing immune systems may, by allocation or by default, achieve greater survival via a high nutritional plane. Indeed, in the Soay sheep, there is some evidence of senescence affecting immune cells such as CD4 T-cells (Nussey *et al.* 2012) which could impair antibody production in older individuals. Finally, older females may rely on Ig subtypes more relevant to nematode-specific immunity, such as IgA or IgE which are increased in older Soay sheep (Watson *et al.* Submitted).

Additional analyses suggest that our conclusions are robust to various potential confounders. Our biological interpretation of the survival analysis using these principal components as predictors was confirmed by analysis using the raw variables, which likewise revealed age-dependence of the nutritional effects (Table S2). The interpretation of relationships between immunological and ecological variables is

312 complicated by the possibility that within-year associations between immune markers can vary with year
313 of observation (e.g. Ardia 2007; Hegemann *et al.* 2012; Pigeon *et al.* 2013). Similarly, nutritional indices
314 are known to vary both seasonally (as exemplified for the fat by Mautz 1978) and annually, depending for
315 instance on the quality and quantity of available forage (Herfindal *et al.* 2006). However, such effects
316 were not present in our case. Using the year of capture as a fixed effect, we checked for significant
317 interactions between the principal components and the year of capture: neither interaction was significant
318 (PC1 x Year of capture: $p = 0.40$; PC2 x Year of capture: $p = 0.63$), while the best model retained a
319 similar structure (albeit with Year of capture as a significant predictor indicating variable survival
320 between years). Storage represents another potential bias likely to create year-to-year variations in long-
321 term studies such as the one presented here. Long-term storage, for example, may result in degradation of
322 proteins, such that lower levels would be found in older samples. However, this is likely not a concern
323 here, as the lowest levels of total proteins in Soay sheep were found in the most recent year sampled (see
324 Figure S2) and given consistency of survival predictors across years.

325 We also did not find associations between protein nutritional markers and parasite burden estimated as
326 FEC. In this population, FEC are highest in lambs (Hayward *et al.* 2014a), on whom parasite-mediated
327 selection is strongest between crashes (Hayward *et al.* 2011). It is thus perhaps unsurprising that we did
328 not detect associations of FEC with malnutrition or survival in ewes. There are, however, reasons to
329 expect nutrition to interact with parasite burdens. For example, when parasites interfere with resource
330 allocation and actively suppress immunity, low resource acquisition rates may result in stagnating
331 immune defense and increased parasite loads (Cressler *et al.* 2014). This 'negative spiral' has been
332 described for naturally helminth-infected humans (e.g. Crompton & Nesheim 2002) and experimentally-
333 infected rodents (Koski & Scott 2001) and proposed as a crucial general framework in disease ecology
334 (Beldomenico & Begon 2010). However, evidence for the negative spiral is scarce in other species, in
335 particular in the wild. This may be because, while sound immunological tests for non-model species are
336 increasingly available (Garnier & Graham 2014), the assessment of the nutritional status of an animal in

337 wild settings remains difficult (Wagner *et al.* 2013). In the context of estimating the costs of immunity,
338 body weight is often used as a marker of condition (Lochmiller & Deerenberg 2000), but is not
339 necessarily an adequate currency to reflect differential investment in different resource compartments
340 such as fat or proteins. Similarly, in ungulates, protein and fat resources are often assessed using
341 "biometric" measures (e.g. Cook *et al.* 2010) which may or may not correlate with serum metabolites
342 (Keech *et al.* 1998). Particularly when these measures are taken from hunted animals (e.g. Adamczewski
343 *et al.* 1997), they pose the significant problem of relating them to individual fitness. As shown here and in
344 other nutritional ecological studies of wildlife (Gilot-Fromont *et al.* 2012; Jego *et al.* 2014), it is possible
345 to adapt techniques from veterinary medicine to specifically assess protein nutrition from a physiological
346 standpoint. In the Soay sheep, detecting a negative spiral would require a longer time series of nutritional
347 and immunological markers including crash and non-crash years as well as all sexes and ages. Interesting
348 information could also be gained from samples collected in early winter, closer to the time of peak
349 mortality. Such samples, at a time when protein reserves should show signs of depletion, may deepen
350 understanding of the trade-off between nutrition and immunity in this system.

351 Parasites, particularly gastrointestinal nematodes on their own (e.g. Hudson *et al.* 1998; Townsend *et al.*
352 2009) or in mixed infections (Ezenwa & Jolles 2015), are expected to be crucial drivers of wild animal
353 population dynamics. However, the relative costs of parasitism and defense that shape the evolution of
354 resource allocation strategies and indeed determine the demographic rates that drive those population
355 dynamics are borne by individual hosts. Empirical quantification requires that suitable currencies are
356 measured and varied condition is taken into account. Here we have provided rare evidence for the much-
357 hypothesized (e.g. Lochmiller & Deerenberg 2000) resource costs of immune defense. Future challenges
358 include discovery of how resistance and tolerance interplay to mitigate the impact of parasitism at the
359 individual level (Athanasiadou *et al.* 2015). Such studies are essential to elucidate the evolutionary and
360 proximate causes of defense heterogeneity in nature.

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555 association with over-winter survival.

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Table legends

Table 1: Variance explained by and correlation loadings of the first three Principal Components (PC1, PC2 and PC3) of a Principal Component Analysis on the nutritional (Total Proteins; Albumin) and immunological (Anti-Nuclear Antibodies, ANA; Anti-*T. circumcincta* IgG) markers.

Table 2: AIC, log likelihood and estimates (± SE) for the best models of winter survival probability of the Soay sheep as a function of the Principal Components (PC1 and PC2), with age being modelled as a class ("AgeClass") or as continuous ("Age"). All models include the year of capture as a random effect ("1|CapYear" following the syntax of the 'lme4' package in R). Weight at the time of capture is also included, after mean centering and scaling to unit variance ("stdWeight").

Figure legends

Figure 1: Positive effect of the first Principle Component (PC1) on overwinter survival of Soay sheep. Age is modeled as an age class. Blue curve represents the mean, and the shaded area around this represents the associated 95% confidence interval. Green dots represent sheep surviving the winter while red dots are for sheep that died in the winter following plasma sampling.

Figure 2: Interaction between the age class (Prime age: 2-6 years old; Old age: 7 years old and older) and the second Principle Component (PC2) on the overwinter survival probability of Soay sheep. The survival probability of younger individuals (blue curve) decreases with PC2, while older individuals with higher values of PC2 survive better (green curve). In both cases, the curve represents the mean effect in the best statistical model, while the shaded areas represent the associated 95% confidence interval. Green dots represent sheep surviving the winter while red dots are for sheep that died in the winter following plasma sampling. Plain dots represent older individuals, open circles represent younger individuals.

583 **Tables**

584

585 Table 1

Principal Component	Variance explained	Correlation loadings			
		Albumin	*Total Proteins*	*Anti-T. circumcincta IgG*	*Anti-Nuclear Antibodies*
PC1	40.94%	0.36	0.77	0.71	0.63
PC2	30.41%	0.83	0.35	-0.32	-0.55
PC3	16.05%	-0.01	0.20	-0.61	0.47

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588 Table 2

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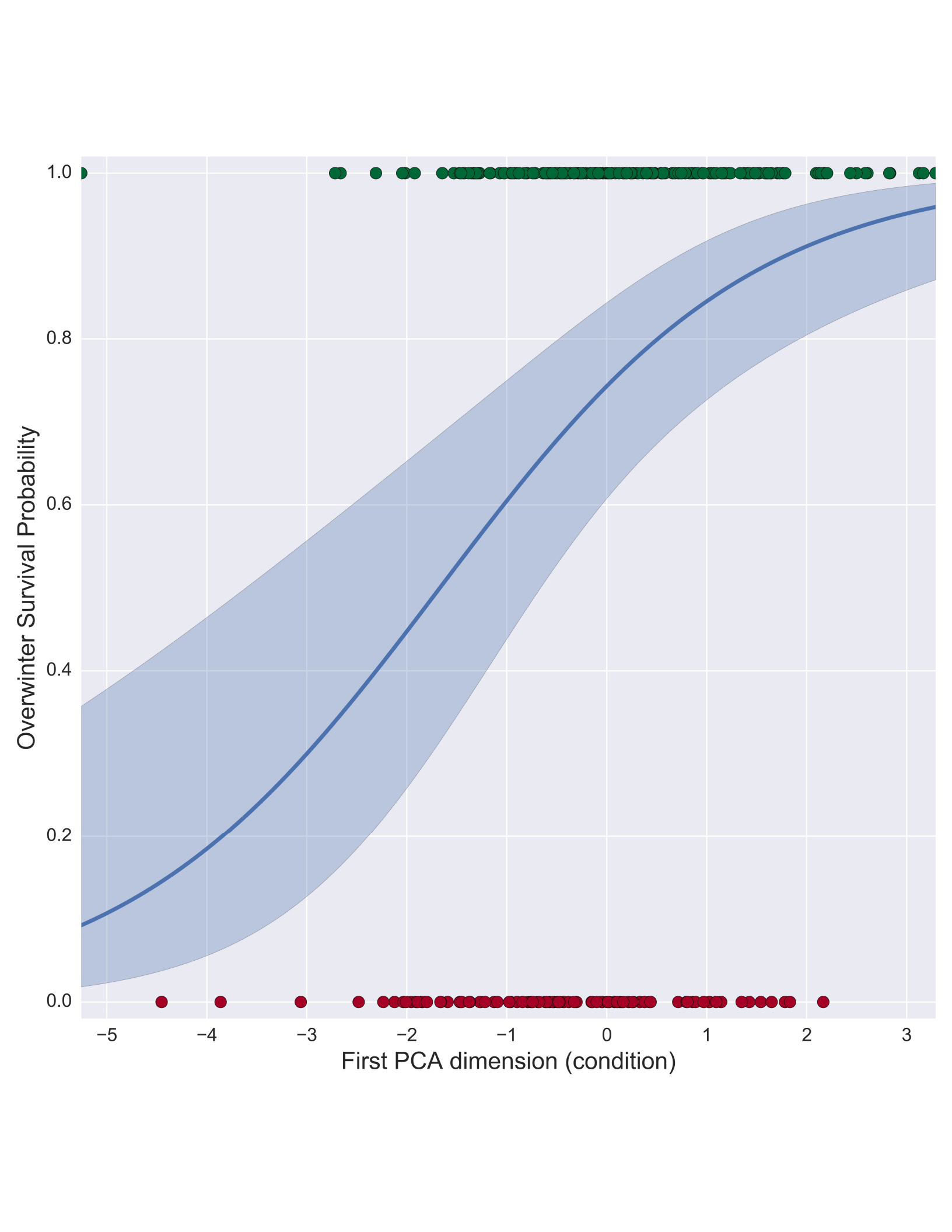
Model structure	AIC	LogLik	Age/ AgeClass - Prime	Weight (standardized)	PC1	PC2	PC2: (Age/ AgeClass - Prime)
With age as age classes (Prime-age and Old individuals)							
AgeClass + stdWeight + (1|CapYear)	315.7	-153.8	2.11±0.35 ($p < 0.001$)	0.67±0.17 ($p < 0.001$)			
AgeClass+stdWeight+PC1+PC2+ PC2:AgeClass+(1|CapYear)	296.2	-141.1	2.07±0.37 ($p < 0.001$)	0.73±0.19 ($p < 0.001$)	0.64±0.16 ($p < 0.001$)	0.36±0.26 ($p = 0.164$)	-0.90±0.32 ($p=0.004$)
With continuous age							
Age + stdWeight + (1|CapYear)	296.9	-144.4	-0.50±0.07 ($p < 0.001$)	0.90±0.19 ($p < 0.001$)			
Age+stdWeight+PC1+PC2 +PC2:Age +(1|CapYear)	282.2	-134.1	-0.48±0.07 ($p < 0.001$)	0.94±0.21 ($p < 0.001$)	0.62±0.16 ($p < 0.001$)	-0.90±0.40 ($p = 0.023$)	0.12±0.06 ($p = 0.048$)


1.0
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Overwinter Survival Probability
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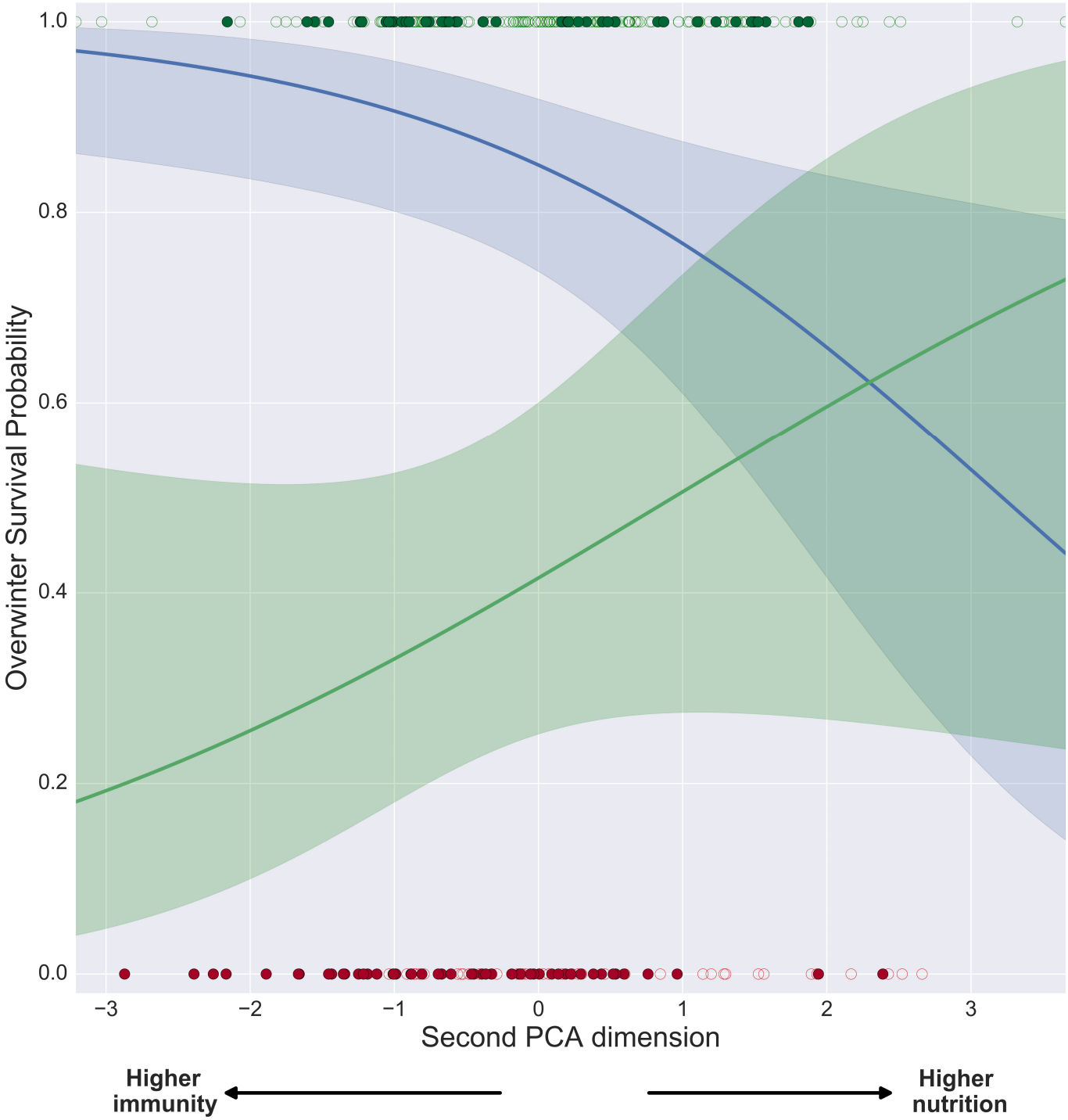
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Overwinter Survival Probability
−3
−2
−1
0
1
2
3
Second PCA dimension
Higher immunity
Higher nutrition

Supplementary information

Figure S1: Correlation circle of the first two Principle Components of the Principal Component Analysis. All four markers cluster on PC1, while PC2 separates nutritional proteins (Albumin, Total Proteins) from antibodies (Anti-Nuclear Antibodies (ANA), Anti *T. circumcincta* IgG).

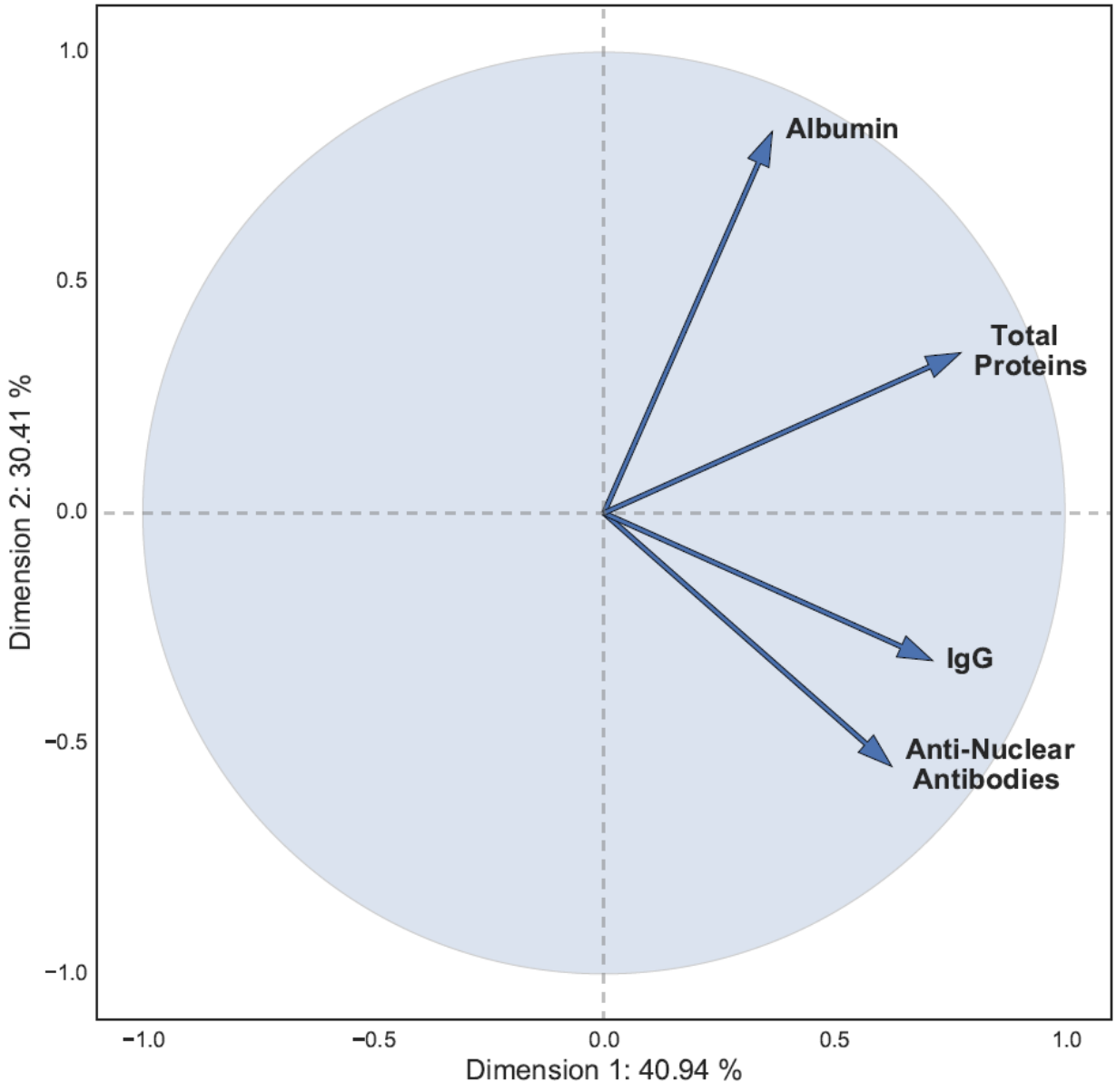

Figure S2: Interannual dynamics of total protein plasmatic levels. We used a linear model to assess the effect of year of capture on the levels of plasmatic TP and found an overall significant effect ($F = 10.80$; $p < 0.001$). Post-hoc analysis showed that levels in 1998 and 2004 were indistinguishable (estimate 2004-1998: -0.06 ± 0.17; $p = 0.91$), but that sheep caught in 2011 had significantly lower levels than sheep caught in 1998 (estimate 2011-1998: -0.63 ± 0.15; $p < 0.001$) or 2004 (estimate 2011-2004: -0.56 ± 0.15; $p < 0.001$). Overall, this pattern is the opposite of what would be expected if proteins had been degraded over long periods of storage. However, it justifies inclusion of total protein in analyses of albumin and immunoglobulin data as well as a random effect of capture year, to ensure that differences among years are taken into account in our multi-year survival analyses. .

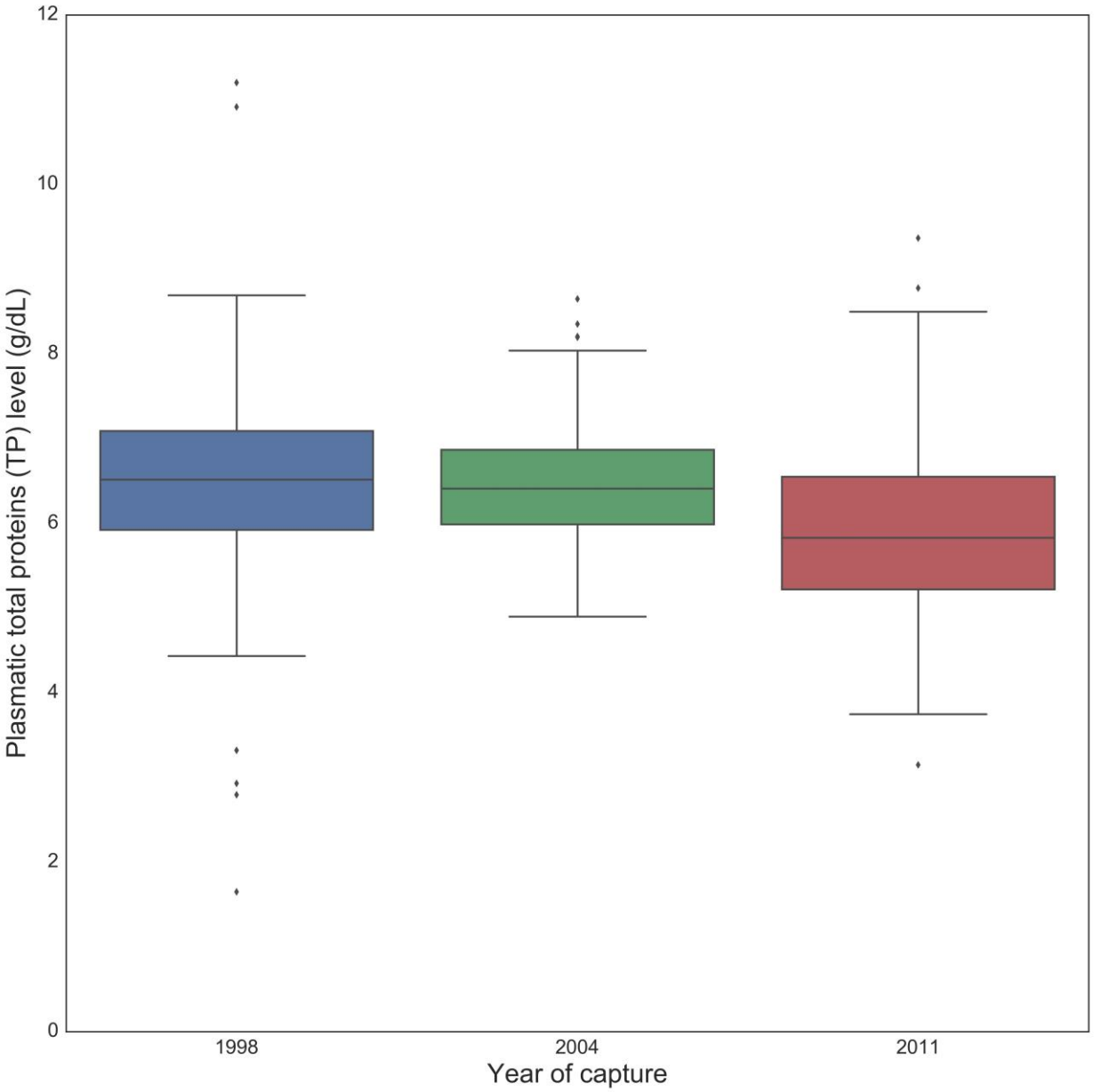

Table S1: Association between the four markers of interest, albumin (Alb), total proteins (TP), anti-nuclear antibodies (ANA) and anti-*T. circumcincta* IgG (IgG Tc). Pearson's r and associated p-values are reported.

	Alb	**TP**	**ANA**	**IgG Tc**
Alb		r = 0.365 p < 0.001	r = -0.110 p = 0.06	r = -0.026 p = 0.66
TP			r = 0.243 p < 0.001	r = 0.292 p < 0.001
ANA				r = 0.350 p < 0.001
IgG Tc				

Table S2: Best logistic regression model for the overwinter survival of Soay sheep as a function of the different markers of interest: albumin (Alb), total proteins (TP), antinuclear antibodies (ANA) and anti-*T. circumcincta* IgG (IgG Tc). Age interactions were tested for all markers, and were never retained for IgG Tc. Model selection was performed according to AIC. For each main effect, the parameter estimates ('est') and its associated p-value are reported. In addition to those reported below, all models include main effects of age class ("AgeClass") and weight (mean centered and scaled to unit variance, "StdWeight") and a random effect for the year of capture ("CapYear" corresponding to 1998, 2004 or 2011). However, because significant effects of AgeClass and stdWeight were already described elsewhere and are not the focus of the current work, the details of their effects are not given here (see Table 2 in the main text for details about these).

Main effects included	AIC	IgG Tc	Alb	Alb: AgeClass (Prime)	TP	TP:AgeClass (Prime)	ANA	ANA: AgeClass (Prime)
IgG Tc + Alb + TP + ANA + TP:AgeClass + ANA:AgeClass	287.5	est = 2.75 p < 0.001	est = 0.57 p = 0.064		est = 0.53 p = 0.110	est = -0.82 p = 0.024	est = -2.71 p = 0.058	est = 4.74 p = 0.014
IgG Tc + TP + ANA + TP:AgeClass + ANA:AgeClass	288.2	est = 2.68 p < 0.001			est = 0.64 p = 0.048	est = -0.76 p = 0.034	est = -2.68 p = 0.071	est = 4.93 p = 0.012
IgG Tc + Alb + Alb:AgeClass	289.0	est = 2.65 p < 0.001	est = 1.12 p = 0.033	est = -0.98 p = 0.114				
IgG Tc + ANA + ANA:AgeClass	289.2	est = 2.74 p < 0.001					est = -1.79 p = 0.19	z = 3.93 p = 0.034